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Oral application of recombinant *Bacillus subtilis* spores to dogs results in a humoral response against specific *Echinococcus granulosus* paramyosin and tropomyosin antigens

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Running Head: Recombinant *B. subtilis* spores for oral immunization

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ABSTRACT

Bacillus subtilis is known as an endospore- and biofilm-forming bacterium with probiotic properties. We have recently developed a method for displaying heterologous proteins on the surface of *B. subtilis* biofilms by introducing the coding sequences of the protein of interest into the bacterial genome to generate a fusion protein linked to the C-terminus of the biofilm matrix protein TasA. Although *B. subtilis* is a regular component of the gut microflora, we constructed a series of recombinant *B. subtilis* strains that were tested for their ability to immunize dogs following oral application of the spores. Specifically, we tested recombinant spores of *B. subtilis* carrying either the fluorescent protein mCherry or else selected antigenic peptides (tropomyosin and paramyosin) from *Echinococcus granulosus,* a zoonotic intestinal tapeworm of dogs and other carnivores. The application of the recombinant *B. subtilis* spores led to the colonization of the gut with recombinant *B. subtilis* but did not cause any adverse effect on the health of the animals. As measured by ELISA and immunoblot, the dogs were able to develop a humoral immune response against mCherry as well as against *E. granulosus* antigenic peptides. Interestingly, the sera of dogs obtained from immunization with recombinant spores of *E. granulosus* peptides were able to recognize *E. granulosus* protoscoleces, which represent the infective form of the head of the tapeworms. These results represent an essential step towards the establishment of *B. subtilis* as an enteric vaccine agent.

INTRODUCTION

The enteric immunization of mammalian species, including canids, is notably challenging. In the particular case of dogs, oral vaccines against parvovirus (1, 2), rabies (3) or canine infectious respiratory disease (4, 5) are both based on live attenuated organisms (virus and bacteria, respectively). Nevertheless, an environmentally safe method of immunization for the delivery of antigens that prevents the shed of the live attenuated pathogen has not been yet established in dogs. Several attempts of carriers for enteric antigens based on a viral or bacterial background have been developed recently, unfortunately with only moderate success (6-9). *Bacillus subtilis* is a well described and versatile microorganism, able to form endospores, develop into biofilms and even used as a probiotic in humans or livestock (10-12). We recently engineered *B.* subtilis for the display of heterologous proteins on the surface of the biofilm. To this end, the matrix protein TasA was fused to various peptides on its C-terminus, and the fusions were expressed in biofilm-inducing conditions (13). To optimize expression, we used the genetic background *tasA/sinR.* On one hand, SinR is a repressor of the *tapA-sipW-tasA* operon and other genes. Therefore, the *sinR* mutant resulted in enhanced expression levels of the fusion protein. On the other hand, the *tasA* mutant background would prevent competition of the endogenous TasA with the fusion protein (14, 15).

An interesting pathogen is the zoonotic canine intestinal cestode *Echinococcus granulosus sensu lato* (16) responsible for causing cystic echinococcosis (CE) in mammalian and marsupial intermediate hosts, but also in humans as accidental hosts. The worldwide-distributed CE (17) is considered one of the most important neglected tropical diseases (18). Thus, CE is responsible for high human morbidity and mortality and is also

64 responsible for significant economic losses in livestock (19). The *E. granulosus* life cycle
65 includes canids (mainly domestic dogs) as definitive hosts, which become infected by
66 ingesting lungs or livers of intermediate hosts rich in *Echinococcus* cysts containing
67 protoscoleces. Once the protoscoleces get in close contact with the intestinal mucosa of
68 dogs, they develop into the parasitic form, i.e., 5-7 mm long, egg-producing tapeworms
69 (16). The eggs of *E. granulosus* are shed in the feces of the definitive host by either the
70 release of the last proglottid in the intestine or the direct excretion of the tapeworms.
71 Intake of these tapeworm eggs by intermediate or susceptible accidental hosts then
72 initiates a new round in the parasite's life cycle. Since dogs are the main host and they
73 spread disease by excreting parasite eggs in their feces, control programs are required that
74 include regular deworming of the dog population (20). A vaccine for immunologically
75 attack the egg-producing tapeworm might greatly help in reducing the risk of disease for
76 humans. In previous studies, two potential antigens were proposed as vaccine targets
77 against *E. granulosus* intestinal infections, namely the proteins tropomyosin (EgTrp) and
78 paramyosin (EgA31) (21-24).
79 In the present study, we investigated the ability of recombinant *B. subtilis*
80 to induce an antibody response in dogs upon oral application of spores containing TasA
81 linked to mCherry or *E. granulosus* peptides (EgTrp and EgA31).
82
83 **RESULTS**
84 **The intestinal microflora of dogs contains *B. subtilis*.** In a first instance, we wondered
85 if the intestinal microflora of dogs could be disturbed by the presence of exogenous *B.*
86 *subtilis*. To answer this question, we first investigated whether *B. subtilis* was part of the

intestinal microflora in healthy dogs. For this purpose, feces were collected from either the University of Zurich (UZH) dog's facility or from privately owned dogs. Each fecal sample was processed as indicated in the scheme depicted in **Figure 1A.** Our data imply that all the isolated bacteria that were able to sporulate and form biofilms in MSgg semi-solid media belonged to *Bacillus spp.* **(Figure 1B and C**). Additionally, the isolated bacteria did not show a significant genetic divergence **(Figure 1B)** from the *B. subtilis* non-domesticated strain, NCIB 3610. Interestingly, we could also find close relatives as *B. vallismortis* and *B. aerophilus*. Taken together, our data indicate that *B. subtilis* is ubiquitous in the intestinal microflora of healthy dogs and that it is likely that the non-domesticated *B. subtilis* NCIB 3610 will not significantly disturb the balance of the intestinal microflora.

Luminescent *B. subtilis* bacteria could be detected in the dog gut. We next wondered whether recombinant spores of *B. subtilis* could germinate in the gut of dogs and display an antigen of interest. For this purpose, we incorporated the *luxCDABE* operon from *Photohabdus luminescens* (25), driven by a *tapA* promoter in a *B. subtilis tasA/sinR* strain harbouring also the *tapA-sipW-tasA-mCherry* operon **(Table 1),** generating the *B. subtilis tasA/sinR/lux*/TasA-mCherry strain. The genetic background *tasA/sinR* in *B. subtilis* improves the expression of heterologous genes driven by the *tapA* promoter (13). We hypothesized that this newly engineered strain would permit the easy tracking of *B. subtilis* by luminescence when in a vegetative state. As expected, an enhanced bioluminescence was observed when comparing the expression of *B. subtilis luxCDABE* (*wt/lux*) with that of *B. subtilis tasA/sinR/lux*/TasA-mCherry biofilms at 24, 48 and 72 h post-inoculation **(Figure 2A).** As observed in **Figure 2B,** such enhancement in

luminescence expression was also improved when the biofilms were incubated at 37˚C instead of 30˚C. Interestingly, already at 24 h post-inoculation, the expression of *B. subtilis tasA/sinR/lux/*TasA-mCherry was significantly increased when incubated at 37˚C rather than at 30˚C. Consistent with our previous research (13), the sporulation ability of *B. subtilis tasA/sinR/lux/*TasA-mCherry strain was decreased compared to *B. subtilis wt/lux* when growing at either 30˚C or 37˚C **(Figure 2C).** As depicted in **Figure 2D** (upper panel), no luminescence was detected in *B. subtilis tasA/sinR/lux/*TasA-mCherry endospores when diluted in milk, possibly since the *tapA* promoter is not active in the endospores. Milk was used as the media for the oral application of recombinant *B. subtilis* spores to dogs. Instead, vegetative cells (germinated from the same spore aliquot) were luminescent (**Figure 2D, lower panel**). The switch from non-luminescence in spores to luminescence in vegetative cells will permit to easily track the germination *in vivo* of the recombinant *B. subtilis tasA/sinR/lux/*TasA-mCherry spores by using an *in vivo* imaging system (IVIS) equipment.

As a proof of principle, we used oral application to inoculate spores from *B. subtilis tasA/sinR/lux/TasA-mCherry* into two-weeks old puppies (**Table 2,** dogs #1 and #2). First, we followed the spores shedding in the puppies' feces during three days (24, 48 and 72 hpa (hours post-application)). For the detection of the recombinant spores, germinated spores in selective LB semi-solid media were initially monitored for luminescence expression. As observed in **Figure 2E**, the bacterial luminescence was detected at 48 hpa in puppy #2 and 72 hpa in both puppies. As the gastrointestinal transit in puppies is close to 24 h (26), our data suggest that the spores were retained in the gut of the animals, and a small fraction was shed three days after (72 hpa).

We next explored whether puppies could elicit a humoral immune response against the TasA-mCherry fusion. For this purpose, we provided the dogs with recombinant spores of the *B. subtilis tasA/sinR/lux*/TasA-mCherry strain on days 21 and 42 after the first application. Interestingly, the dogs elicited a specific humoral immune response of both types IgG and IgA against the two tested recombinant proteins (H_6-mCherry **(Figure S1, A, and C)** and H_6-TasA **(Figure S1, B, and D)**. At day 60, the different intestinal sections (duodenum, jejunum, ileum/cecum, and colon) were inspected for luminescence using an IVIS equipment **(Figure 2F)**. The monitoring of the opened intestine sections evidenced luminescence mainly in the jejunum, ileum/cecum, and colon but not in the duodenum. Consistent with this observation, we could also determine the presence of our recombinant bacteria by immunohistochemistry against TasA (anti-TasA) and mCherry (anti-dsRed2) (**Figure S1E**). Our results indicate that recombinant spores of *B. subtilis tasA/sinR/lux/*TasA-mCherry can germinate in the gut and get established in the intestinal microflora, permitting the heterologous expression of both Lux and TasA-mCherry proteins.

Recombinant spores of *B. subtilis* induce a specific humoral response against TasA-mCherry in dogs. We next administrated dogs with recombinant spores of *B. subtilis* by starting the applications in nine-weeks-old animals, which is in agreement with the Swiss schedule for dog's vaccination. To this end, we included three dogs in our experiments, separated in one group of two dogs (**Table 2,** dogs #4 and #5) orally applied with recombinant *B. subtilis tasA/sinR/lux/*TasA-mCherry spores and a second group composed by a single dog, orally applied with milk (placebo) (**Table 2,** dog #3). The animals received three doses of recombinant *B. subtilis* spores on days 1, 21 and 42. The

elicitation of a systemic humoral immune response against the fusion TasA-mCherry was monitored by indirect ELISA using weekly-collected sera samples. Only dogs orally applied with recombinant *B. subtilis* spores were able to elicit a specific humoral immune response against H_6-mCherry **(Figures 3A and S2A)**. This response was more pronounced for the dog #4 (grey bars). Remarkably, this humoral immune response consists of IgG but not of IgA isotype **(Figure S2A)** In contrast, indirect ELISA against the non-related antigen glutathione S-transferase (GST) did not show any measurable response from the dog's sera, either for IgG or IgA antibody isotypes **(Figure 3B and Figure S2B).** Consistent with our data, we could also observe a specific H_6-mCherry humoral immune response by immunoblotting. Indeed, we also found an antibody response of IgG isotype **(Figure 3D)** and not of IgA isotype **(Figure S2D)** when checking for sera at day 60. Interestingly, we could observe that all the tested animals (inoculated with recombinant *B. subtilis* spores or placebo) were able to respond against H_6-TasA protein for both antibody isotypes, IgA and IgG **(Figure 3C and Figure S2C)**.

We further investigated if the recombinant spores get shed in dog's feces after each application. For this purpose, anal swabs were collected daily up to six days post-application after the first and second oral application (on days 1 and 21) of recombinant *B. subtilis* spores (**Table 3**), cultivated on selective solid media, and the grown colonies were later examined in an IVIS imaging system for the detection of *luxCDABE* operon luminescence. Only dog #4 shed recombinant spores on the second day after the first and second oral application. For the third oral application of recombinant *B. subtilis* spores (**Table 4**), feces were collected daily in a random manner since the Swiss animal experimental rules prohibit separate caging of dogs. Under these conditions, three random

179 samples (RS) were collected daily during three days post-application. The presence of
180 recombinant *B. subtilis* spores in feces was confirmed by germination in selective LB
181 semi-solid media, followed by *luxCDABE* luminescence detection and further
182 quantification of the positive colonies. Our data shows that RS feces contain spores on
183 days 1 to 3 following the third oral application. Interestingly, and consistent with our
184 experimentation schedule, only two feces samples contained recombinant *B. subtilis*
185 spores on day 1 (RS1 and RS2) and day 2 (RS1 and RS3) suggesting that these samples
186 were from the two dogs of the recombinant *B. subtilis* spore group. Then on day 3,
187 however, only one feces sample contained recombinant *B. subtilis* spores (RS2). On day
188 60 **(Figure 3F)**, different intestinal sections were inspected, and inflammatory cells were
189 not found either in placebo or in dogs orally inoculated with the recombinant *B. subtilis*
190 spores. This result indicates that the recombinant *B. subtilis* spores, or the vegetative cells,
191 do not have any detrimental effect on the intestinal structures. This finding is consistent
192 with the probiotic abilities of *B. subtilis* (27). Collectively, our data show that dogs orally
193 applied with recombinant *B. subtilis tasA/sinR/lux/*TasA-mCherry spores can elicit a
194 specific humoral response against the TasA-mCherry fusion.
195 **Induction of intestinal immunity in dogs against *E. granulosus* antigens**. Next, we
196 wondered whether a specific humoral immune response against the integumental antigens
197 from the parasite *E. granulosus* (EgTrp and EgA31) could be elicited by using the
198 immunization methodology described above. For this purpose, we used two different
199 recombinant *B. subtilis* spores, engineered to express the fusion proteins TasA-(102-207)
200 EgTrp or TasA-(370-583)EgA31 (**Table 1** and (13)). For simplification, we hereafter
201 refer to these as *B. subtilis* TasA-EgTrp and *B. subtilis* TasA-EgA31. As described by

202 Vogt *et al*., 2016 (13), these strains can display the *E. granulosus* antigenic peptides
203 (EgTrp and EgA31) fused in frame to the C-terminus of TasA on the surface of *in vitro*
204 biofilms. For this experiment, four groups of two dogs each (**Table 2**) were employed.
205 Three groups were orally applied on days 1, 21 and 42 with recombinant *B. subtilis*
206 spores harbouring fusions of TasA-EgTrp (group EgTrp), TasA-EgA31 (group EgA31)
207 and a mixture of both recombinant spores (TasA-Egtrp and TasA-EgA31) (group
208 mixture). The fourth group was the placebo control, which received only milk. The
209 animals were nine weeks old when they received the first oral application. In agreement
210 with our previous results, no variations in the body weight between of all the
211 experimental groups were observed during the whole procedure (**Figure S3A**). The
212 elicitation of the humoral response for the TasA fused to *E. granulosus* antigens was
213 monitored weekly from collected sera of the treated dogs. For this purpose, an indirect
214 ELISA was developed for the detection of specific antibodies against the purified
215 proteins H_6-EgTrp, H_6-EgA31, H_6-TasA and the non-related antigen H_6-mCherry. Of
216 note, maternal antibodies can be detected in young dogs as an early signal (7-15 days
217 post-immunization) in ELISA (28) and therefore, was considered as not significant. We
218 noticed that at least one of the dogs in each of the groups receiving a single kind of
219 recombinant *B. subtilis* spores showed to be positive for its respective antigen (dog#2 of
220 group Tas-EgTrp (black bars) and dog#2 of group TasA-EgA31 (black bars)), when
221 tested for specific IgG antibodies against H_6-EgTrp **(Figure 4A)** and H_6-EgA31 **(Figure**
222 **4B).** However, the dogs that received the mixture of recombinant spores responded
223 mainly to H_6-EgA31, especially dog#2 (mixture group, black bars). Placebo dogs did not
224 react to any of these two antigens **(Figures 4 A and B)**. None of the animals responded

against the non-related H_6-mCherry antigen **(Figure 4C)**, showing the specific nature of the immune response mounted by the recombinant spores. Consistent with our previous result (**Figure 3**) all the animals presented a specific immune response against H_6-TasA (**Figure 4D**). However, when testing the sera for specific IgA antibodies (**Figures S3 B-E**) targeting H_6-Egtrp, H_6-EgA31, H_6-mCherry, and H_6-TasA, no immune response was detected for any of these antigens.

We next questioned if the sera of the dogs collected on day 60 could also recognize the antigens in the parasite context. The two antigens used in this study, EgA31 and EgTrp, have been described previously as localizing in the tegument and subtegument outer membranes of the *E. granulosus* protoscolex (23, 29, 30). For this purpose, immunohistochemistry was performed over *E. granulosus* protoscoleces by incubation with the dog sera. For the analysis of the data, we took particular consideration of the staining of the subtegument/tegument membranes, corresponding to the enlarged insets shown in **Figure 4E.** The brownish coloration observed in the internal regions of the protoscoleces by immunohistochemistry was considered as background because it is not possible to distinguish between incubation with placebo and spores-treated sera. Our results reveal that the subtegument/tegument membranes presented brown coloration (inset, black arrowheads) when incubated with sera of dogs #2 of group EgTrp and group EgA31 and for both dogs of group mixture. In contrast, the sera of placebo dogs, dog #1 of the EgTrp group and dog #1 EgA31 group revealed a negative coloration (blue) of subtegument/tegument membranes of the protoscoleces. These data are consistent with the immune response observed by ELISA (**Figures 4 A-D**) and

247 strongly suggest that the dogs treated with recombinant *B. subtilis* spores harbouring *E.*
248 *granulosus* antigens could recognize the parasite.

249 In an attempt to re-isolate the applied recombinant *B. subtilis* from the gut of the
250 treated animals, we recovered the intestinal microflora of duodenum, jejunum, and ileum
251 of two dogs, which received the recombinant *B. subtilis* spore mixtures. The recombinant
252 *B. subtilis* were isolated in selective media (**Table 1**). Interestingly, recombinant *B.*
253 *subtilis* bacteria could be separated from both the duodenum and the jejunum of the dog
254 #2, but no recombinant bacteria were isolated from the intestine of dog #1. The presence
255 of recombinant bacteria was confirmed by PCR of colonies isolated from duodenum and
256 jejunum of dog #2 from the mixture group (**Figure 4F**).

257 Importantly, neither inflammatory nor degenerative lesions could be detected by
258 histology in the intestinal sections. Likewise, no undesirable clinical signs were observed
259 in the dogs after treatment with spores (**Figure S3F**). These data confirm that the use of
260 recombinant spores from *B. subtilis* is safe for dogs. Our results indicate that oral
261 application of recombinant *B. subtilis* spores harbouring TasA fusions to *E. granulosus*
262 antigens (EgTrp and EgA31) get germinated in the gut and elicited a specific humoral
263 response in dogs.

264

265 **DISCUSSION**

266 Current evidence indicates that *B. subtilis* endospores can germinate in the gut of
267 mice, rabbit, and humans (31, 32). Consequently, engineered *B. subtilis* spores may be
268 used as carriers for the administration of antigens in the immunization against enteric
269 pathogens such as viruses, bacteria, and parasites or even for the induction of tolerance in

the treatment of chronic inflammatory diseases (33). In this study, we provide evidence that oral application of recombinant *B. subtilis* spores in dogs resulted in spore germination in the gut. The presence of vegetative recombinant bacteria could favour the formation of an intestinal biofilm, allowing the stimulation of the gut-associated lymphoid tissue (GALT) (31), which elicited a humoral response against TasA fused to an epitope. In fact, our results show that a humoral immune reponse in dogs against the fusion protein TasA-mCherry, and tapeworm *E. granulosus* fusions TasA-EgTrp and TasA-EgA31 was mounted, suggesting spore germination and GALT stimulation by the recombinant *B. subtilis* spores.

In contrast to other methodologies based on surface-expression or in surface-adsorption of antigens on spores of *B. subtilis* (34), our approach consisted in the expression of heterologous peptides after spore germination. This method is particularly advantageous for oral application since the spores can bypass the stomach barrier without damaging the structure of the heterologous peptide. Another advantage of using *B. subtilis* as an antigen carrier is its probiotic nature (27, 35) that permits a safe application. We demonstrated that there was no significant difference in body weight of the dogs during the whole procedure as well as no undesirable clinical signs in the animals receiving recombinant *B. subtilis* spores. Hematoxylin/eosin-stained histological cuts from different intestinal sections showed neither inflammatory nor degenerative lesions after the oral application of the recombinant *B. subtilis* spores. The fact that *B. subtilis*, as showed here and by others (36, 37), is part of the dog's natural intestinal microflora, reinforces the safety of this organism for its use in the delivery of antigens in the intestine. Altogether, our data show that *B. subtilis* can safely deliver heterologous peptides in the

intestine of dogs to subsequently mount an immune response. Interestingly, and in line with our results, the biofilm formation ability of *B. subtilis* strains isolated from intestinal microflora has also been described in species as diverse as grass carp (38) or human (39, 40). It has been suggested that the biofilm-forming properties of *B. subtilis* strains are relevant for the growth and formation of biofilms in the intestine (32, 40). In this study, we used the non-domesticated *B. subtilis* NCIB 3610 strain to engineer recombinant spores. This strain has been extensively studied for its remarkable biofilm-forming abilities (15). Thus, the recombinant spores used in this study (**Figure 2** and (13)) have the potential to germinate and then form a biofilm in the mucosa of the intestine. We present strong evidence indicating that recombinant *B. subtilis* colonize and express the heterologous proteins in the dog's intestine as denoted by: i) the visualization of vegetative recombinant *B. subtilis* by luminescence (**Figure 2F**), immunohistochemistry against the specific antigens **(Figure S1E)** and specific PCR of perfused intestinal content **(Figure 4F)**; ii) the quantification of recombinant *B. subtilis* spores shed in feces, which are in lower numbers than the initial load of spores suggesting an intestinal retention and germination of the spores **(Tables 3 and 4)**.

By using indirect ELISA and immunoblotting with dog sera, we demonstrated that dogs generated a humoral response against the presented antigens, mCherry or *E. granulosus* peptides. Interestingly, the tested dogs also developed a humoral response against TasA, which was also positive for placebo dogs in all the experimental settings. This result suggests that native TasA derived from the dog's intestinal microflora could stimulate local immunity, which relates to the maturation of the intestinal microflora of the dogs. It has been proposed by others (41) that the generation of antibodies against the

intestinal microflora promotes the host-microbiota mutualism through a reduction of the inflammatory response towards the microbiota, allowing the immune exclusion of the bacteria in direct contact with the intestinal mucosa.

Even if the humoral response recognizes the recombinant *E. granulosus* antigens EgA31 and EgTrp, further research will unveil whether this technology can neutralize parasitic infections. A good indication in this direction is the recognition of the tegument and subtegument from the isolated *E. granulosus* protoscoleces by the serum of the recombinant *B. subtilis* spores-treated dogs.

Collectively, our results demonstrate that the oral application of recombinant *B. subtilis* spores elicited a specific humoral response in dogs. This technology could be the foundation for the development of vaccines for dogs that carry *B. subtilis* in their intestinal microbiota.

MATERIALS AND METHODS

Ethics statement. All the dog experiments were performed according to the guidelines of the animal experimentation law (SR 455.163; TVV) of the Swiss Federal Government. The Cantonal Veterinary Office of Zurich approved the protocols with the following animal experimentation number 100/2010.

***B. subtilis* strains, media and culture conditions.** A list with the *B. subtilis* strains used in this study is shown in **Table 1**. For routine growth and spore quantification, cells were propagated on Luria-Bertani (LB) medium. For biofilm assays, cells were scraped from overnight growth on LB-agar plates, resuspended in LB liquid medium to an $OD_{600\ nm}$ of 1, and then 2 µl of this suspension were spotted on MSgg solid medium (42). Biofilms

were incubated at 30°C or 37˚C. The final concentration of antibiotics used for the *B. subtilis* strains were the following: spectinomycin (Spc) (100 µg/ml), kanamycin (Km) (10 µg/ml), MLS: erythromycin (1 µg/ml) and lincomycin (25 µg/ml), and chloramphenicol (Cm) (5 µg/ml).

Plasmid constructions. pQE80L-EgA31*Pst*I/*Dra*I was obtained from Dr. A-F. Pétavy (Université Claude Bernard Lyon 1, Lyon, France) (43). pQE32-(102-278)EgTrp was achieved by PCR amplification of (102-278)EgTrp fragments from the constructs pQIA-EgTrp, kindly provided by Dr. Adriana Esteves (Universidad de la República, Montevideo, Uruguay) (21) using specific primers containing flanking *BamH*I and *Pst*I restriction sites followed by ligation between *BamH*I and *Pst*I in pQE-32 (QIAGEN). pQE32-mCherry was obtained by PCR amplification of mCherry from pRSET-mCherry (44) using specific primers containing *BamH*I and *Hind*III restriction sites followed by ligation between *BamH*I and *Hind*III in pQE-32 (QIAGEN). pET22b-TasA (45) was provided by Dr. Diego Romero (University of Malaga, Spain). pDR-P_{tapA}-*luxCDABE* was obtained by PCR amplification of *luxABE* genes from pSB403 (25) using specific primers containing *Age*I and *Sac*I restriction sites, followed by ligation between *Age*I and *Sac*I in pDR183-P_{tapA}/*luxA*. The plasmid pDR183-P_{tapA}/*luxA* was obtained by PCR amplification of *luxCDA* genes from pSB403 using specific primers to insert at the 5' end an *EcoR*I restriction site followed by RBS (ribosome binding site) and at the 3' end *AgeI*/*Sac*I restriction sites. The PCR fragment was ligated between *EcoR*I and *Sac*I in pDR183-P_{tapA}. The plasmid pDR183-P_{tapA} was obtained by PCR amplification of P_{tapA} from pBS-TapAop-mCherry (13) using specific primers containing *Sac*II and *EcoR*I restriction sites, followed by ligation between *Sac*II and *EcoR*I in pDR183 [*lacA*:: erm] (46) (kindly

provided by Dr. David Rudner, Harvard University, MA, USA). All oligonucleotides were obtained from Microsynth AG, Switzerland and described in **Table S1**.

Antibodies and reagents Rabbit polyclonal anti-TasA was a gift from Dr. R. Losick (Harvard University, Cambridge, USA). Mouse polyclonal anti-EgTrp and mouse polyclonal anti-EgA31 were a gift from Dr. M-F. Pétavy (Université Claude Bernard Lyon 1, Lyon, France). Goat anti-GST was purchased from GE Healthcare Life Sciences. Mouse anti-dsRed2 was purchased from Santa Cruz Biotechnology, USA. Rabbit F(ab')2 anti-dog IgG H&L-peroxidase was acquired from Abcam. Goat anti-dog IgA antibody-peroxidase was obtained from Bethyl Laboratories, Inc. (Montgomery, TX, USA). Rabbit anti-goat IgG (whole molecule)-peroxidase and Goat anti-mouse IgG(Fab)'-peroxidase were obtained from Sigma-Aldrich. Isopropyl-ß-D-thiogalactopyranoside (IPTG) was acquired from Biosolve Chimie, France.

Transformation of *B. subtilis*. *Bacillus subtilis* strain 168 was transformed as described previously by Cutting and Vander Horn (47). The transformants were selected with the appropriate antibiotics for a double crossover recombination at the *amyE* locus or *lacA* locus (48). The different operons were then transferred to undomesticated *B. subtilis* NCIB 3610 strain by SPP1-mediated generalized transduction (49). The positive clones were identified by direct PCR of the selected colonies using specific primers.

Characterization and identification of *B. subtilis* in the dog intestinal microflora. Feces were analyzed from seven samples (21 dogs) of dogs from the UZH animal facility and nine individual samples collected from privately owned dogs. All the samples were from healthy animals that had not been treated for at least one year with antibiotics or probiotics. The feces were resuspended in ten times weight volume with PBS, serially

385 diluted, plated in LB (Luria-Bertani) agar media and incubated for two days at 37˚C. The
386 isolated colonies were streaked in Difco sporulation agar media for 24 h at 37˚C and
387 selected for rod shape by microscope inspection using a 63X lens. Then, the selected
388 isolates were re-inoculated in 3 ml of Difco sporulation media, incubated on an orbital
389 shaker for four days at 37˚C and inspected at the microscope for the presence of
390 endospores. The selected sporulating rod bacteria were stored at -80˚C in 15% glycerol
391 for further analysis. The sequence of the *rrnE* gene of 16S was analyzed as described by
392 Hoa *et al.*, 2000 (50). Briefly, PCR amplification was performed using primers specific
393 for *B. subtilis rrnE* gene of the 16S rRNA (P1, 5'-GCGGCGTGCCTAATACATGC-3'
394 and P2, 5'-CACCTTCCGATACGGCTACC-3') and then sequenced by the Sanger
395 method at Microsynth, AG, Switzerland. Sequences were aligned with CLUSTALX (51).
396 The optimal model of DNA evolution was evaluated for best fit of the dataset using
397 MRAIC (Nylander, J. A. A. 2004. MrAIC.pl. Program distributed by the author;
398 Evolutionary Biology Centre, Uppsala University). Bayesian phylogeny was inferred
399 using BEAST (version 1.5.3). Markov Chain Monte Carlo simulation with GTR
400 substitution matrix and a strict clock was run over 10'000'000 generations. The tree files
401 were combined into one consensus tree by using LOGCOMBINER. The consensus tree
402 was displayed with FIGTREE (52), representing the similarities between the 16S rRNA
403 genes. The *rrnE* gene sequence from undomesticated *B. subtilis* NCIB 3610 strain was
404 used as a reference. A list of the assigned bacteria strain from the isolated clones is
405 provided in **Table S2.**

406 **Quantification of spores in a biofilm.** The ability of recombinant *B. subtilis* to sporulate
407 in a biofilm was determined as described by Vlamakis *et al.* (15). Briefly, *B. subtilis*

cultured in LB media was diluted to an $OD_{600\ nm}$ of 1; and 10 µl of the suspension was inoculated in duplicate over 2.5 ml of MSgg medium in 12-well culture plates. The culture plates were incubated at room temperature with no agitation. Samples of cells were taken after 48 h and subjected to mild sonication conditions (10 s at 14 kHz) to obtain intact single cells. After sonication, each preparation was normalized to $OD_{600\ nm}$ of 1, incubated for 20 min at 80 °C to kill vegetative cells and serially diluted to determine viable spore counts in selective LB-agar plates.

Production of recombinant *B. subtilis* spores. The recombinant spores were produced and purified as described by Vogt *et al.*, 2016 (13). A list of the *B. subtilis* strains used in this study is provided in **Table 1**.

Expression and purification of His_{x6} tagged proteins. pQE31-mCherry, pQE32-(102-278)EgTrp and pQE80L-EgA31PstI/DraI were expressed in *Escherichia coli* (*E. coli*) M15[pREP4] (Qiagen). pET22b-TasA was expressed in *E. coli* BL21 (New England BioLabs, Inc). The culture was induced with isopropyl β-D-thiogalactoside (1 mM), grown for 4h, and centrifuged at 3,500 rpm for 15 min. The pellet was resuspended in 6 ml of PBS and incubated for 15 min on ice with 0.1 mg/ml lysozyme, 5 mM DTT, 1.5% lauryl sarcosine and cOmplete™ protease inhibitor cocktail (Roche, Switzerland). The lysate was sonicated (six times for 10 s each) and centrifuged at 12,000 x g for 15 min. The supernatant was supplemented with 1% Triton X-100 and loaded onto a Ni Sepharose 6 Fast Flow (GE Healthcare), previously equilibrated with five volumes of 20 mM imidazole in 50 mM NaH_2PO_4, for 2h at 4˚C in a rotation wheel. Subsequently, the resin was washed with 10 volumes of 60 mM imidazole, 200 mM NaCl, 0.1% Triton X-100 and 50 mM NaH_2PO_4, then with 10 volumes of 60 mM imidazole, 300 mM NaCl,

431 0.1% Triton X-100 and 50 mM NaH_2PO_4 and finally with 10 volumes of 60 mM
432 imidazole, 400 mM NaCl, 0.1% Triton X-100 and 50 mM NaH_2PO_4. The protein was
433 eluted four times by incubating 10 min each with 250 µl of 250 mM imidazole, 400 mM
434 NaCl, 0.1% Triton X-100, and 50 mM NaH_2PO_4.

435 The GST protein was expressed from pGEX-6P-1 (GE Healthcare) in *E. coli*
436 BL21 and purified following the instructions provided by the manufacturer.

437 The eluted proteins were pooled in a single fraction and stored at 4˚C in 30%
438 glycerol. The protein concentration was obtained by comparison to a BSA calibration
439 curve in SDS-polyacrylamide gel followed by Coomassie blue staining. The estimation of
440 the protein concentration was performed by densitometry using the gels algorithm from
441 ImageJ 1.48v software (Wayne Rasband, NIH, USA).

442 **Biofilm Imaging.** Whole colonies were photographed, and the images were processed as
443 described by Vogt *et al*., 2016 (13).

444 **Experimental administration of recombinant *B. subtilis* spores in dogs.** In the
445 Vetsuisse animal facility of the University of Zurich, 2-4 beagle dogs were housed in
446 pens of a surface of 1.45 m x 4.5 m with access to an outside area of 1.45 m x 5.5 m. Also,
447 pens were enriched with installations permitting dogs to play in a three-dimensional
448 space, to rest and to retreat. Beagle dogs were raised for laboratory use in the Vetsuisse
449 animal facility of the University of Zurich. All the dogs were vaccinated against
450 leptospirosis, canine distemper, canine hepatitis, parvovirus infection and parainfluenza
451 viruses (Canigen L, Virbac, and Canigen, SHA_2PPi). The dogs were orally applied three
452 times on days 1, 21 and 42 with 5 x 10^{10} CFU per dose of recombinant *B. subtilis* spores.
453 Each dose of the recombinant *B. subtilis* spores was diluted in 1 ml of cat milk

(Whiskas® Mars, Virginia USA) to increase palatability. The placebo group received 1 ml of cat milk. Each oral gavage was performed in morning hours after feeding the dogs. All the animals were clinically monitored in weekly routine and every day after each oral administration. Data on the animal number assigned, sex, age, and kind of recombinant *B. subtilis* spore applied to each dog are provided in **Table 2.** Blood samples (6 to 8 ml) were collected from cephalic vein weekly, starting one day before the first oral gavage. The serum was used to test humoral response against purified *E. coli* recombinant proteins.

Animal necropsy. The dogs were sacrificed with an intravenous administration of acepromazine 0.1 ml/10 kg (Prequillan™, AROVET AG, Switzerland) followed by three doses of 80 mg/kg of sodium pentobarbitone (Esconarkon, Streuli Pharma AG, Switzerland). During the post-mortem examination blood sample, feces and intestinal sections were collected.

Immunoblotting. Identical amounts of purified recombinant protein (3 µg) were mixed with sample buffer (8 % SDS, 40 % Glycerol, 200 mM Tris pH 6.8, 4 % 2-mercaptoethanol, 0.4 % Bromophenol blue), heated for 5 min at 95˚C and loaded consecutively in a 12% SDS-polyacrylamide gel. After migration, the proteins were transferred to an Amersham™ Protan™ 0.45 µm nitrocellulose blotting membrane (GE Healthcare) and lanes were cut into stripes. The stripes were incubated in dogs sera diluted (1:100) in 5% milk-PBS. Samples were further processed as described by Glück *et al*. (53).

Indirect ELISA. Recombinant purified protein (500 ng/well) in 0.2 M bicarbonate buffer pH 9.4 was coated for 16 h at 4˚C in a 96 well multi-well plates (Nunc-Immuno Maxisorp,

ThermoScientific). Sample wells were incubated for 2 h at room temperature with blocking buffer (5% milk, 0.1% Tween-20 in PBS). The dog's sera were diluted to 1:500 in blocking buffer, added 50µl per well and incubated overnight at 4˚C. Samples were incubated with the corresponding secondary anti-dogs antibody conjugated to HRP diluted in blocking buffer for 1 h at 37˚C in a moist chamber. Between each incubation, the plates were washed three times with PBS containing 0.05% Tween-20. The ELISA was developed by the addition of 100 µl per well of TMB substrate (ThermoFisher Scientific) in the dark for 30 min at room temperature, and the reaction was stopped by adding 100µl 1M H_2SO_4. The plate's absorbance was read at $OD_{450\,nm}$ using an SLT 340 ATTC Tecan microplate reader (Tecan US Inc.). The data were analyzed and processed using Microsoft®Excel® for Mac 2011. The cut-off was determined as the average of three negative controls. The negative control value was obtained by incubation of the antigen only with the secondary antibody conjugated to HRP. The cut-off was subtracted from all the sample values. Each value has been subtracted to its corresponding pre-immune value (PI).

Histology and immunohistochemistry. The dog's intestines were sectioned in duodenum, jejunum, ileum, cecum, and colon. Each intestinal section was knotted in both ends previous to sectioning to avoid the loss of the intestinal content. The samples of approximately 2 cm length were fixed in 4% formaldehyde. After fixation, each sample was dehydrated in alcohol solutions of increasing concentration and embedded in paraffin. The embedded samples were cut at 2-3 µm thick slides and stained with hematoxylin and eosin (HE).

For immunohistochemistry of the dog's intestinal section, the samples were de-

paraffinized, rehydrated and incubated for 30 min at room temperature with the primary antibody (rabbit anti-TasA serum or mouse anti-dsRed2 serum). A detection kit, containing the secondary antibody and aminoethyl carbazole (AEC) as chromogen, was subsequently applied according to the manufacturer's protocols (Peroxidase/AEC Rabbit/Mouse Kit, DAKO).

E. granulosus protoscoleces were isolated from a sheep liver cyst. The samples were treated for immunohistochemistry by fixing, embedding in paraffin and de-paraffinizing as described above. Subsequently, the samples in EDTA buffer pH 9.0 (1.25 mM EDTA, 10mM Tris pH 9.0) were heated using a steamer (Pascal, Dako Cytomation) for 20 min at 98˚C. The slides were blocked for 10 min at room temperature with Peroxidase-Blocking Solution, Dako REAL (#S2023, DAKO). The samples were then incubated with the indicated dog serum (diluted 1:50) followed by a rabbit F(ab')2 anti-dog IgG (H&L) conjugated to HRP (1:100, ab136759, Abcam), stained with liquid DAB+ substrate chromogen system (brown, DAKO) and counterstained with hematoxylin (blue).

Images were acquired using light microscope Olympus CX41 equipped with a 40 X objective lens and Olympus Vanox-S AxioCam interface. The acquired images were processed using Image J software (Wayne Rasband, NIH, USA. http://imagej.nih.gov/ij).

Detection of recombinant spores in intestinal content The small intestine was dissected in duodenum, jejunum, and ileum. Perfusion collected the intestinal content of each section with vigorous flushes of PBS, centrifuged at 14,000 x g and 20°C for 15 min. The pellet was resuspended in 20 ml PBS and centrifuged at 1,500 x g for 10 min at 20˚C. The supernatant was collected, centrifuged at 14,000 x g for 15 min and 20˚C and the

final pellet was resuspended in 3 ml of PBS. Serial dilutions prepared from $1x10^{-1}$ to $1x10^{-6}$, and 10 µl of each dilution plated on semi-solid LB-kanamycin plates (10 µg/ml) and incubated for 36 h at 37°C. The colonies were counted, and the amount of CFU/ml was estimated (15).

Colony PCR. A colony was resuspended in 15 µl lysis buffer (50 mM KCl, 0.1 % Tween 20, 10 mM Tris-HCl pH 8.3) and heated to 99°C for 10 min. The PCR amplicons were obtained by thermocycling 5 µl of bacterial lysate with 15 µl of PCR master mix (0.2 mM dNTPs, 150 ng of each forward and reverse primers, 0.3 µl Taq DNA polymerase (5U/µl, New England Biolabs, Inc.), 1µl DMSO). The following internal primers were used for detection *B. subtilis* recombinant strains; for Egtrp 5'-ATGCG CGGCCGCCATTATGATGGCAATGAAATTG-3' and 5'-GATCCCCGGG GGGATCCTTACTCTTGCTCGGAGACTTCGAG-3', and for EgA31 5'-ATGCGC GGCCGCCGCAGCTGAAAAACAAGCCATG-3' and 5'-GATCCCCGGGGGA TCCTCACCTTGTTTCAAGCATTTCAAT-3'. The PCR amplicons were monitored by agarose gel electrophoresis. Images were acquired using Molecular Imager® Gel Doc™ XR with an incorporated Image Lab™ Software (Bio-Rad) and processed using Image J 1.48v software (W. Rasband, NIH, USA) and Microsoft Powerpoint for Mac 2011.

IVIS. The luminescence of biofilm and intestinal samples were acquired using a Xenogen IVIS™100 Imaging System (Imaging Technologies) equipped with a CDD array scientific camera (Spectral Instruments, Inc.), mounted over light-tight specimen chamber, a 600 series camera controller and a camera cooling system. The images were analyzed and processed using a Living Image, version 4.0 software (Caliper Life Sciences, Inc).

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AVAILABILITY OF DATA AND MATERIAL

The data sets supporting the conclusion of this article are included in the article and its additional files.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments CA MA PD CE. Performed the experiments: CMV MTAF MH CA CE. Analyzed the data CMV MTAF KT MH CA MA PD CE. Contributed reagents/materials/analysis tools: CMV MTAF KT MH CA PD MA CE. Wrote the paper: KT CA MA PD CE.

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FIGURES LEGENDS

Figure 1. *Bacillus subtilis* is present in the dog's intestinal microflora. A) Schematic representation of the procedure used to sequence the 16S *rrnE* gene from sporulating bacteria isolated from feces of dogs of the UZH animal facility and privately owned dogs. **B)** Baysian phylogeny of the 16S *rrnE* gene isolated from sporulating bacteria isolated from privately owned and UZH facility dog's feces samples. Sequences are compared with the reference sequence obtained from undomesticated *B. subtilis* NCIB 3610 (top). Alignment gaps and missing data were eliminated in pairwise sequence comparisons. Bar, 0.0030 changes per nucleotide position. **C)** Colony top view of bacterial clones isolated from dogs feces of the UZH animal facility. Bacteria were growth over MSgg solid media for 72 h at 30˚C. The assigned bacteria clone numbers are denoted at each picture up-left corner. Scale bar is 1 cm.

Figure 2. Tracking of luminescent *Bacillus subtilis* in the gut of dogs. A) Top view of luminescent biofilms from *B. subtilis* strains wt/*lux*, and *tasA/sinR/lux*/TasA-mCherry

743 incubated in MSgg semi-solid media for 24, 48, and 72h at 30˚C. A color luminescence
744 scale is shown for each row. The scale bar is 1 cm. **B)** Comparison of luminescence of *B.*
745 *subtilis* wt/lux (gray bars) and *tasA/sinR/lux*/TasA-mCherry (white bars) biofilms at 30˚C
746 and 37˚C when incubated for 24, 48 and 72h. The data represent the mean ± SEM (t-test,
747 ** $p<0.01$, n=4). **C)** Sporulation ability of *B. subtilis wt/lux,* and *tasA/sinR/lux/*TasA-
748 mCherry of 72h biofilms when incubated at 30˚C and 37˚C. The data represent the mean
749 ± SEM (t-test, * $p<0.05$ and ** $p<0.01$, n=4). **D)** Comparison of the luminescence of
750 spores resuspended in milk (upper panel) and vegetative cells (lower panel) from *B.*
751 *subtilis tasA/sinR/lux*/TasA-mCherry strain. A color luminescence scale is shown for each
752 panel. **E)** Germination of spores isolated from anal swabs of dogs #1 and #2 after the first
753 oral application of the *B. subtilis tasA/sinR/lux*/TasA-mCherry spores. Anal swabs
754 samples were collected at 48 and 72 hpa. The spores were incubated in selective LB
755 semi-solid medium and monitored for luminescence of vegetative cells. **F)** Detection of
756 luminescence from *B. subtilis tasA/sinR/lux*/TasA-mCherry vegetative cells in the gut of
757 dogs #1 and #2, after oral application of three doses of recombinant *B. subtilis* spores
758 ($5x10^{10}$ CFU on days 1, 21 and 42). Each picture corresponds to open portions of
759 representative sections of the duodenum, jejunum, ileum/cecum, and colon of each
760 animal. A color luminescence scale is shown for each picture. All luminescent images
761 were acquired with a Xenogen IVIS camera and analyzed using Living Image® 4.0
762 software (Caliper Life Sciences, USA).

763 **Figure 3. Immune response after oral application in dogs of recombinant *Bacillus***
764 ***subtilis tasA/sinR/lux*/TasA-mCherry spores.** Dogs sera, at the indicated days post-
765 application, were tested by indirect ELISA using plates coated with *E. coli* purified H_6-

mCherry **(A)**, GST **(B)** and H_6-TasA **(C)** and followed by incubation with specific anti-IgG dog-HRP. The grey, white and black bars correspond to dogs #4, #5 and placebo, respectively. Dogs #4 and #5 were orally applied with recombinant *Bacillus subtilis tasA/sinR/lux/TasA-mCherry* spores. Placebo dog received only milk. The data represent the mean ± SEM of three independent experiments. **D)** Detection of *E.coli* purified H_6-mCherry by immunoblotting test stripes incubated with the indicated dog serum (diluted 1:100) at day 60 post-application followed by incubation with specific anti-dog whole IgG-HRP. The positive control (+) (lane 1) was incubated with a specific mouse anti-dsRed2 followed by anti-mouse-HRP. The arrow indicates the position of mCherry. **E)** Histological sections stained with hematoxylin and eosin from intestinal samples of dogs orally inoculated with recombinant *B. subtilis* spores (dogs #4 and #5) or placebo dog. Lm, intestinal lumen. Scale bar is 100µm.

Figure 4. A specific humoral response is elicited in dogs after oral application of recombinant *B. subtilis* spores harbouring *Echinococcus granulosus* antigens. Dog's sera, at the indicated days post-application, were tested by indirect ELISA using plates coated with *E. coli* purified H_6-EgTrp **(A)**, H_6-EgA31 **(B)**, H_6-mCherry **(C)** and H_6-TasA **(D)**. The samples were then incubated with a specific anti-IgG dog-HRP. The dog groups are indicated on the vertical axis. White and black bars correspond to the dogs #1 and #2 of each group, respectively. The data represent the mean ± SEM of three independent experiments. **E)** Immunohistochemistry of sheep *E. granulosus* protoscoleces incubated with the indicated dogs sera (1:100) followed with secondary anti-IgG dog-HRP and stained with diaminobenzidine (brown). Nuclei were counterstained with hematoxylin (blue). Each row corresponds to the indicated the dog group (placebo, EgTrp, EgA31, and

789 mixture). The orally applied dog number is reported at the top of each panel. The right
790 columns of the two panels correspond to enlargement insets of the indicated black frame
791 at the left picture. The black arrows show the positive sera recognition of the
792 protoscoleces subtegument/tegument membrane. Scale bar is 50 µm. **F)** PCR
793 characterization of intestinal recombinant *B. subtilis* bacteria isolated from the small
794 intestine of dog #2 from mixture group. Isolated colonies of one duodenum (D) and two
795 jejuna (J1 and J2) sections were analyzed by PCR for the detection of *B. subtilis*
796 *tasA/sinR/*TasA-EgTrp (upper panel) and *B. subtilis tasA/sinR/*TasA-EgTrp (lower panel)
797 strains, respectively. In each panel, also included a set of controls namely EgTrp, EgA31
798 and ΔTΔS which correspond to PCRs laboratory *B. subtilis tasA/sinR/*Tas-EgTrp, *B.*
799 *subtilis tasA/sinR/*Tas-EgA31 and *B. subtilis tasA/sinR* strains, respectively. The yellow
800 arrows indicated the position of each specific band.

801

802 **TABLES**

803 TABLE 1. *B. subtilis* strains used in this study.

Strain	Genotype[a]	Reference/source
wt/*lux*	*lacA::*P_{tasA}*-luxCDABE; Erm*^{R}	This study
*tasA/sinR/lux/*TasA-mCherry	*tasA::Km*^{R}*;SinR::Spc*^{R}*; lacA::*P_{tasA}*-luxCDABE; MLS*^{R}*; AmyE::yqxM-sipW-tasA-mCherry ; Cm*^{R}	This study
tasA/sinR	*tasA-sinR::Km*^{R}	Vogt *et al.*, 2016 (13)
*tasA/sinR/*TasA-(102-207)EgTrp	*tasA-sinR::Km*^{R}*; amyE::yqxM-sipW-tasA-(102-207)EgTrp; Spc*^{R}	Vogt *et al.*, 2016 (13)
*tasA/sinR/*TasA-(370-583)EgA31	*tasA-sinR::Km*^{R}*; amyE::yqxM-sipW-tasA-(370-583)EgA31; Spc*^{R}	Vogt *et al.*, 2016 (13)

[a] Km^R, kanamycin resistance gene; Spc^R, spectinomycin resistance gene; Cm^R, chloramphenicol resistance gene; MLS^R, erythromycin and lincomycin gene resistances.

TABLE 2. Dog identification number, gender, age and type of *B. subtilis* spore orally applied.

Animal number	Gender[e]	Age (weeks)[a]	*B. subtilis* spore strain[b]
1,2	M, M	2	*tasA/sinR/lux/*TasA-mCherry
3	F	9	Placebo[c]
4, 5	M, F	9	*tasA/sinR/lux/*TasA-mCherry
1, 2	F, M	9	Placebo[c]
1, 2	M, F	9	*tasA/sinR/*TasA-(102-207)EgTrp
1, 2	M, F	9	*tasA/sinR/*TasA-(370-583)EgA31
1, 2	M, F	9	Mixture[d]

[a] Age corresponding to the first *B. subtilis* spores received.

[b] $5x10^{10}$ CFU per dose of orally applied recombinant *B. subtilis* spores.

[c] Milk

[d] $2.5x10^{10}$ CFU *tasA/sinR/*TasA-(102-207)EgTrp and 2.5 $x10^{10}$ CFU *tasA/sinR/*TasA(370-583)EgA31 spores per dose.

[e] F, female, and M, male.

TABLE 3. Summary of the presence of recombinant spores of *B. subtilis tasA/sinR/lux/*TasA-mCherry strain in feces of dogs after the first and second oral applications.

	First oral application			Second oral application		
DPA[a]	[#4][b]	[#5]	placebo	[#4]	[#5]	placebo
1[c]	-	-	-	-	-	-
2	+[d]	-	-	+	-	-
3	-	-	-	-	-	-

4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-

[a] DPA, days post-application

[b] Dogs #4 and #5 were treated with $5x10^{10}$ CFU spores/dose of *B. subtilis* tasA/sinR/lux/TasA-mCherry strain diluted in milk and placebo was treated with milk only.

[c] Samples obtained by anal swabs.

[d]The samples were considered positive (+) when resistant to heat treatment (80˚C for 20 min) and displaying luminescence after germination, as inspected with an IVIS camera.

TABLE 4. Presence and quantification of *B. subtilis tasA/sinR/lux/TasA-mCherry* spores in feces after a third oral application.

DPA[a]	1		2		3	
RS[b]	luminescence[c]	spores[d]	luminescence	spores	luminescence	spores
1	+	$2.4x10^5$	+	$3.0x10^6$	-	nd
2	+	$6.5x10^5$	-	nd	+	$1.3x10^4$
3	-	nd	+	$1.7x10^5$	_	nd

[a]DPA, days post-application, and [b]RS, random feces sample.

[c]The samples were considered positive (+) when resistant to heat treatment (80˚C for 20 min) and displaying luminescence after germination, as inspected with an IVIS camera.

[d]The number of recombinant *B. subtilis* spores is represented as the average of two independent measurements. The data corresponds to CFU per gram of feces. nd, not detected.